Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for the Titration of Newcastle Disease Vaccine, Infectious Bronchitis Vaccine, and Combination Newcastle Disease-Infectious Bronchitis Vaccine in Chicken Embryos

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1. Introduction

This Supplement Assay Method (SAM) describes a procedure for titrating Newcastle disease (ND) vaccine, Infectious Bronchitis (IB) vaccine, and combination ND-IB vaccine. The vaccines are reconstituted and inoculated into embryonated chicken eggs in 10-fold dilutions such that the 50% endpoint of infectivity (EID_{50}) can be calculated directly on a per field dose basis by the Reed-Muench method.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Centrifuge (Beckman J-6B, JS-4.2 rotor)

2.1.2 Humidified, rotating egg incubator (Midwest Incubators, Model 252)

2.1.3 Vortex mixer (Thermolyne Maxi Mix II Model No. M37615)

2.1.4 Pipette (Rainin Pipetman, P1000)

2.1.5 Cool-lite tester (Val-A)

2.1.6 Egg candling light on stand (Speed King)

2.1.7 Etcher electric engraver (Vibro-graver Acme Burgess, Inc.)

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 Cotton swabs/cotton balls

2.2.2 Serological pipets (Falcon, Cat. No. 7530)

2.2.3 Specific-pathogen-free (SPF) chick embryos, 9- to 11-day-old

2.2.4 Disposable sterile filters, 0.45 *u*m (Millex-HA Millipore)

2.2.5 Pipette tips (Rainin Clean-Pak disposable microliter pipette tips RT-200)

2.2.6 Syringe, glaspak, 1-cc tuberculin, frosted tip, single use (Becton, Dickinson and Company)

2.2.7 Hypodermic needle, 18 gaugex1½ in (Becton, Dickinson and Company, PrecisionGlide needle)

2.2.8 Hypodermic needle, 25 gaugex⅝ in (Becton, Dickinson and Company, PrecisionGlide needle)

2.2.9 Glass test tubes, 16x125 with morten closures

2.2.10 Glass test tubes, 13x100 with morten closures

2.2.11 Duco cement

2.2.12 Pipette tips (Rainin 0-100, 0-200, 100-1000 or equivalent)

2.2.13 Solutions

1. Tryptose phosphate broth (TPB)

TPB	29.5 g
q.s. with distilled or deionized water	1000.0 ml

Sterilize by autoclaving

2. Penicillin/Streptomycin (pen/strep)

penicillin g	15.775 g
streptomycin	100.0 g
q.s. with distilled or deionized water	1000.0 ml

3. Normal saline (0.85%)

NaCl	8.5	g
q.s. with distilled or deionized water	1000.0	ml

Sterilize by autoclaving

4. 70% alcohol

ethyl alcohol	70.0	ml
q.s. with distilled or deionized water	30.0	ml

5. Iodine 2% in alcohol

iodine	2.0	g
ethyl alcohol (70%)	100.0	ml

3. Preparation for the test

3.1 Personnel qualifications/training

The executor must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. The executor must also have knowledge of safe operating procedures and policies and Quality Assurance (QA) guidelines of the Center for Veterinary Biologics-Laboratory (CVB-L) or equivalent; and training in the operation of the necessary laboratory equipment listed in part **2.1**.

3.2 Preparation of equipment/instrumentation

Operate all equipment/instrumentation according to manufacturer’s instructions and monitor in compliance with current corresponding CVB-L/National Veterinary Services Laboratories Standard Operating Procedures (SOPs), or equivalent.

3.3 Preparation of reagents/control procedures

Prepare reference viruses in the same manner as sample preparation.

3.4 Preparation of the sample

3.4.1 ND vaccine

1. Reconstitute ND vaccine, frozen or lyophilized, in 4°C tryptose phosphate broth (TPB) to a total vol as listed in **Table 1**. Vigorously mix for a minimum of 30 sec, and hold in an ice bath for 15 min to allow for virus disaggregation. Mix again and further dilute the reconstituted vaccine in TPB to obtain the 10^{0} concentration (1 dose per 0.1 ml) as shown in **Table 1**.

2. Make 10-fold dilutions of the 10^{0} virus concentration, 10^{-1} through 10^{-8}, by serially mixing 0.5 ml of the virus with 4.5 ml of TPB plus antibiotics (500-u penicillin and 2.0-mg streptomycin per ml TPB). Use a separate pipette for each transfer. Mix each dilution well before proceeding to the next dilution, and keep all dilutions on ice.

3.4.2 IB vaccine

Prepare the same as for the ND vaccine (See **3.4.1**), making 10-fold dilutions through 10^{-6}.

3.4.3 Combination ND-IB vaccine

Prepare the same as for the ND vaccine. For titration of the Newcastle disease virus (NDV) fraction, the dilutions are inoculated without further treatment. For titration of the infectious bronchitis virus (IBV) fraction, neutralize the NDV by adding 0.8 ml of each dilution, 10^{-2} through 10^{-6}, to an equal vol (0.8 ml) of anti-NDV serum. Mix well, and incubate in an ice bath for 30 min prior to inoculation.

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Table 1

Number of doses in 1 vial	Reconstituted Volume of Vaccine	Additional dilution for 10 concentration
100	10.0 ml	none
500	50.0 ml	none
1,000	50.0 ml	10.0ml vaccine plus 10.0ml diluent
2,000	50.0 ml	3.4 ml vaccine plus 10.0ml diluent
2,500	50.0 ml	2.5 ml vaccine plus 10.0ml diluent
5,000	100.0 ml	2.5 ml vaccine plus 10.0ml diluent
10,000	100.0 ml	1.2 ml vaccine plus 10.0ml diluent
25,000	100.0 ml	2.1 ml vaccine plus 50.0ml diluent

4. Performance of the test

4.1 Egg inoculation

Prior to reconstituting the vaccine, prepare and label the appropriate number of eggs for allantoic cavity inoculation.

4.1.1 NDV inoculation

Inoculate the 10^{-4} through 10^{-8} virus dilutions of the ND or ND-IB vaccine. Use 5, 9- to 11-day-old embryonated chick eggs per dilution (25 eggs total), and inoculate 0.1 ml of the appropriate dilution per embryo.

4.1.2 IBV inoculation

Inoculate the 10^{-2} through 10^{-6} dilutions of the IB or the NDV-neutralized ND-IB vaccines. Use 6, 9- to 11-day-old embryonated chick eggs (30 eggs total). Inoculate 0.1 ml of the appropriate dilution of the IB vaccine or 0.2 ml of the appropriate dilution of the NDV-neutralized ND-IB vaccine per embryo.

4.2 Incubation

Incubate the eggs for 7 days candling daily. Deaths occurring the first 24 hr shall be considered due to trauma and not used in calculations. At least 4 embryos per dilution must be viable at 24 hr postinoculation (PI) for a valid test.

5. Interpretation of the test results

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5.1 Controls

Titrate a known positive reference virus with each group of titrations. The titer of the positive reference virus must be within the established range for the test results to be valid.

5.2 NDV

Record all deaths occurring after 24 hr as positive. On the seventh day PI, open all remaining eggs and examine the embryos. All obviously stunted embryos are considered positive.

5.3 IBV

Record all deaths occurring after 24 hr as positive. On the seventh day PI, open all remaining eggs and examine the embryos for IBV lesions. An embryo exhibiting 1 or more lesions is considered positive.

1. Massachusetts type

Check for stunting, curling, and clubbed down.

2. Other types

Check for stunting, curling, and clubbed down. Open the embryos and check for bile stasis (dark green liver) and kidney urates.

5.4 Calculations

Determine the log 10 EID_{50} titer using the method of Reed and Muench. This dilution and inoculation procedure allow for a direct readout on a per dose basis. Round to 1 decimal.

5.5 Retests

Conduct retests as required by the Code of Federal Redgulations, Title 9, Part 113.8 (b) and requirements of minimum release in firm's current Outline of Production, Part V.

5.6 Evaluation of test results

5.5.1 The 9CFR 113.8 (b) defines the criteria for a satisfactory/unsatisfactory serial.

5.5.2 The firm's requirements of minimum release/stability titers for each vaccine are listed in the current Outline of Production, Part V, for the specific product code.

6. Report of test results

Titers are reported out as EID_{50} per bird dose.

7. References

1. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493-497.

2. This document was rewritten to meet the current CVB-L QA SAM format. No significant changes were made from the previous protocol. This document supersedes the September 1, 1985, version.